

In the Specification:

Please amend the specification as follows:

Please delete the Title of the Invention and insert in place thereof:

--IMMUNOGENIC COMPOSITIONS COMPRISING DAL/DAT
DOUBLE MUTANT, AUXOTROPHIC ATTENUATED STRAINS OF LISTERIA
AND THEIR METHODS OF USE--

Please add the following section to page 1 of the application:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of co-pending U.S. Application No. 10/136,253, filed May 1, 2002, now allowed, which is a divisional of U.S. Application No. 09/520,207, filed March 7, 2000, now issued as U.S. Patent 6,504,020, which is a divisional of U.S. Application No. 08/972,902, filed November 18, 1997, now U.S. Patent 6,099,848.

Please delete the section titled Brief Description of the Drawings and insert in place thereof:

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, comprising Figures 1A through 1C, is the DNA sequence of the *L. monocytogenes* alanine racemase gene (*dal*) of *L. monocytogenes* (SEQ ID NO:1) and the amino acid sequence encoded thereby (SEQ ID NO:2). The lysyl residue involved in the binding of pyridoxal-P is indicated by an asterisk.

Figure 2, comprising Figures 2A and 2B, depicts the linear alignment of the deduced amino acid sequences of the alanine racemases of *L. monocytogenes* (LMDAL) (SEQ ID NO:2), *B. stearothermophilus*, (BSTDAL) (SEQ ID NO: 3), and *B. subtilis* (BSUBDAL) (SEQ ID NO:4). Identical amino acids are boxed.

Figure 3, comprising Figures 3A through 3C, is the DNA sequence of the *L. monocytogenes* D-amino acid aminotransferase gene (*dat*) (SEQ ID NO:5) and the amino acid sequence encoded thereby (SEQ ID NO:6). The lysyl residue involved in the binding of pyridoxal-P is indicated by an asterisk.

Figure 4, comprising Figures 4A and 4B, depicts the linear alignment of the deduced amino acid sequences of the D-amino acid aminotransferases of *L. monocytogenes* (LMDAT) (SEQ ID NO:5), *S. haemolyticus* (SHAEDAT) (SEQ ID NO:7), *B. sphaericus* (BSPHDAT) (SEQ ID NO:8), and *Bacillus* species YM-1 (BSPDAT) (SEQ ID NO:9). Identical amino acids are boxed.

Figure 5 is a graph depicting the growth requirement for D-alanine of the *dal⁻dat⁻* double mutant strain of *L. monocytogenes*. The *dal⁻dat⁻* (daldat) and wild-type (*L. monocytogenes*+) strains of *L. monocytogenes* were grown in liquid culture in BHI medium at 37°C in the presence (+D-ala) or absence (-D-ala) of exogenous D-alanine (100 µg/ml). In additional experiments, the mutant cell culture was also provided D-alanine after 30 minutes and after 60 minutes.

Figure 6, comprising Figures 6A through 6C, is a series of images of light micrographs depicting the growth of wild-type *L. monocytogenes* (Panel-A) (Figure 6A) and the *dal⁻dat⁻* double mutant strain of *L. monocytogenes* (Panel-B) (Figure 6B) in J774 macrophages at 5 hours after infection with about 5 bacteria per mouse cell. Panel-C Figure 6C illustrates an infection by double mutant bacteria in the continuous presence of D-alanine (80 µg/ml). Arrowheads point to some mutant bacteria.

Figure 7, comprising Figures 7A through 7C, is a series of graphs depicting infection of mammalian cells with the *dal⁻dat⁻* double mutant (open circles) and wild-type strains of *L. monocytogenes* (closed circles). Mammalian cells which were infected included J774 murine macrophage-like cells (Panel-A) (Figure 7A), primary murine bone marrow macrophages (Panel-B) (Figure 7B), and human epithelial cells (HeLa) (Panel-C) (Figure 7C). Panel-A Figure 7A also depicts mutant infection in the presence of D-alanine (100 µg/ml) (closed squares) and in the presence of D-alanine from 0 to 4 hrs during infection (open squares).

Figure 8, comprising Figure 8A through Figure 8J, is a series of images of photomicrographs depicting the association of actin with intracytoplasmic wild-type *L. monocytogenes* ([Panel A] Figures 8A and 8F: 2 hours; Panel-B Figures 8B and 8G: 5 hours) or with the *dal⁻dat⁻* double mutant of *L. monocytogenes* (Panel-C Figures 8C

and 8H: 2 hours wherein D-alanine was present from 0 to 30 minutes; ~~Panel D~~ Figures 8D and 8I: 5 hours, wherein D-alanine was present from 0 to 30 minutes; ~~Panel E~~ Figures 8E and 8J: 5 hours, wherein D-alanine was present continuously), following infection of J744 cells with these bacteria. The images on the top row illustrate the binding of FITC-labeled anti-Listerial antibodies to total bacteria (Figures 8A through 8E), while the bottom row illustrates the binding of TRITC-labeled phalloidin to actin (Figures 8F through 8J). The arrowheads point to some bacteria associated with actin.

Figure 9 is a graph depicting the protection of BALB/c mice against challenge with ten times the LD₅₀ of wild-type *L. monocytogenes* by immunization with the *dal⁻dat⁻* double mutant strain of *L. monocytogenes*. Groups of 5 mice were immunized with the following organisms: (1) 4×10^2 wild-type *L. monocytogenes*, (2) 2×10^7 *dal⁻dat⁻* (+D-alanine supplement), (3) 2×10^5 *dal⁻dat⁻* (+D-alanine supplement), (4) 2×10^4 *dal⁻dat⁻* (+D-alanine supplement), (5) 2×10^2 *dal⁻dat⁻* mutant *dal⁻dat⁻* (no D-alanine supplement). Mice were challenged 21-28 days after immunization. Log₁₀ protection was calculated as described in the Examples.

Figure 10 is a graph depicting the recovery of bacteria from spleens of BALB/c mice following sublethal infection with wild type *L. monocytogenes* (closed circles), the *dal⁻dat⁻* mutant in the absence of D-alanine (open circles), and the *dal⁻dat⁻* mutant in the presence of 20 mg D-alanine (open squares). The points at day 0 illustrate the total number of organisms injected, not the number of bacteria per spleen.

Figure 11, comprising Figures 11A and 11B, is a series of graphs depicting the cytolytic activity of splenocytes isolated from mice at 10-14 days after infection with in Figure 11A, wild type *L. monocytogenes* (●○), or naive control (■□). Figure 11B, *dal⁻dat⁻* mutant: 3×10^7 bacteria (△); 3×10^7 bacteria with boost at 10 days (△); 3×10^7 bacteria wherein animals were provided D-alanine subcutaneously (●○); 3×10^7 bacteria plus 2 mg/ml D-alanine (■) or 0.2 mg/ml D-alanine in drinking water (▲).

Please delete the paragraph beginning on Page 21, line 9, and ending on Page 21, line 24, and insert in place thereof:

The invention should not be construed as being limited solely to the DNA and amino acid sequences shown in Figures 1 and 3. Once armed with the present invention, it is readily apparent to one skilled in the art that any other DNA and encoded amino acid sequence of the *dal* and *dat* genes of other *Listeria* species may be obtained by following the procedures described herein. The invention should therefore be construed to include any and all *dal* and *dat* DNA sequence and corresponding amino acid sequence, having substantial homology to the *dal* and *dat* DNA sequence, and the corresponding amino acid sequence, shown in Figures 1 and 3, respectively (SEQ ID NOS:1, 2, 5, and 6, respectively). Preferably, DNA which is substantially homologous is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous and most preferably about 90% homologous to the *dal* or *dat* DNA sequence shown in Figures 1 and 3, respectively. Preferably, an amino acid sequence which is substantially homologous is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous and most preferably about 90% homologous to the amino acid sequences encoded by the *dal* and *dat* genes shown in Figures 1 and 3, respectively (SEQ ID NOS:1, 2, 5, and 6, respectively).

Please delete the paragraph beginning on Page 15, line 27, and ending on Page 16, line 7, and insert in place thereof:

A cytotoxic T-cell response in a mammal is defined as the generation of cytotoxic T-cells capable of detectably lysing cells presenting an antigen against which the T cell response is directed. Preferably, within the context of the present invention, the T cell response is directed against a heterologous antigen expressed in an AA strain of *Listeria* or which is expressed by a vector which is delivered to a cell via *Listeria* infection. Assays for a cytotoxic T-cell response are well known in the art and include,

for example, a chromium release assay (Frankel et al., 1995, J. Immunol. 155:4775-4782). In addition to a chromium release assay, an assay for released lactic acid dehydrogenase may be performed using a ~~Cytotox-96~~ CYTOTOX™ (non-radioactive cytotoxicity assay 96 kit obtained from Promega Biotech, WI.

Please delete the paragraph beginning on Page 25, line 17, and ending on Page 25, line 23, and insert in place thereof:

Immunohistochemistry. Coverslips with attached infected macrophages or HeLa cells were washed with PBS, and the cells were fixed in 3.2% formalin and permeabilized using 0.05% ~~Tween-20~~ TWEEN 20™(polyoxyethylene (20) sorbitan monolaurate. *Listeria* were detected using rabbit anti-*Listeria* O antiserum (Difco Laboratories) followed by LSRSC-labeled donkey anti-rabbit antibodies or coumarin-labeled goat anti-rabbit antibodies. Actin was detected using FITC- or TRITC-labeled phalloidin. To distinguish extracellular (or phagosomal) from intracytoplasmic bacteria, the former were stained prior to permeabilization of the cells.